REMARKS/ARGUMENTS

This is in response to the Office Action mailed September 21, 2005 for the above-captioned application. Reconsideration and further examination are respectfully requested.

Claims 1-3, 5, 12, 13, 16, 25, 26, 28-30 and 32 were examined.

The Examiner again objected to the amendment filed September 8, 2003 as introducing new matter. Without conceding that the Examiner is correct, Applicants have amended this statement to change the word "including" to the originally-present word "spanning." This should overcome the new matter issue.

The Examiner also maintained the rejection under 35 USC § 112, first paragraph, for lack of written description. Applicants again traverse this rejection.

The Examiner argues that Capon v Eshhar is different from the case at hand because "the claims are not directed simply to a genus of 'cytoplasmic domains', which is admittedly already known in the field, but to any member of a genus of cytoplasmic domains of any of a variety of structurally and functionally disparate 'molecules'. This statement makes no sense. The Examiner admits that a genus of cytoplasmic domains is known, and then turns around and says that the apparently narrower genus of the claim, namely "the cytoplasmic domain of a molecule which functions as a transducer of a mammalian immune response in the presence of a costimulatory factor" is not known.

Furthermore, the Examiner argues that these "disparate molecules and their cytoplasmic domains having this functional property, are **not** limited to those that are *already* known, but instead are reasonably expected to include novel or otherwise poorly characterized molecules having cytoplasmic domains..." This statement with respect to the scope of the claim is true, but it has nothing to do with a proper written description requirement. Applicants invention here (as was the case in *Capon*) is the combined construct. In response, Applicants direct the Examiner's atttention to *In re Fuetterer*, 319 F.2d 259, 138 USPQ 217 (CCPA 1963). The claims in Fuetterer referred to a rubber stock composition useful in producing tire treads and included a functional recitation of "an inorganic salt capable" of maintaining an homogeneous distribution of another component in the composition. The disclosure listed the function desired and four members of the class having that function. The CCPA found that this claim met the requirements of 35 U.S.C. § 112, first paragraph, stating that:

> Appellant's invention is the combination claimed and not the discovery that certain inorganic salts have colloid suspending properties. We see nothing in patent law which requires appellant to discover which of all those salts have such

> properties and which will function properly in his combination. The invention description clearly indicates that any inorganic salt which has such properties is usable in his combination. If others in the future discover what inorganic salts additional to those enumerated do have such properties, it is clear appellant will have no control over them per se, and equally clear his claims should not be so restricted that they can be avoided merely by using some inorganic salt not named by appellant in his disclosure.

319 F.2d at 265, 138 USPQ at 223.

In the present case, ff someone discovers a new cytoplasmic domain, that would be their invention. However, if they put that new domain into a construct within the scope of the present claims that would be using Applicants' invention and it would be wrong to use the written description requirement to prevent claims sufficient to actually cover Applicants' invention as the Examiner is now proposing. The rejection for lack of written description should therefore be withdrawn.

The Examiner again has rejected the claims under consideration as obvious over various new combinations of references. Each of these rejections share as a common basis a reliance on a combination of US Patent Publication 2003/0077249 or WO97/23613 in view of US Patent No. 5,538,866. In his response, he states that a copy of the Guest et al reference was not provided. Applicants now attach another copy. However, it is pointed out that the reference was filed as an attachment to the amendment refiled by facsimile on June 30, 2005, and that it is in the image file wrapper for this date under the heading of "NPL document." Thus, the reference has been part of the record for more than a year, and therefore is properly considered by the Examiner.

It is further noted that the Examiner's argument of what is obvious continues to be far broader than the claims now pending. Whether or not these are at most obvious to try (rather than legally obvious) it is the claimed invention, and not some genus which the Examiner must show to be suggested by the art. Here, the claimed invention requires the selection of a particular combination of scFV (one target to PSMA) and a CD8 hinge. While the Examiner has found all of the pieces, he has found nothing that suggests this particular combination.

A previously stated, the t the art as it relates to fusion proteins with an antibody and a cytoplasmic domain is not limited to the primary references now cited. Other references have been cited earlier which offer teachings of other antibodies, without a CD8 connector. This means that fairly stated, claim 1 of this application is only reached if one skilled in the art makes a particular selection (PSMA) from the antibody column which fairly contains all known antibodies of therapeutic interest, **and** a particular selection from the connector column which contains multiple entries for combination with a cytoplasmic domain. As was the case in *In re*

*Geiger,* at best "one skilled in the art might find it obvious to try various combinations of these known" antibodies and connectors. 12 USPQ2d 1276, 1278 (Fed. Cir. 1987). Obvious- to-try, however, this is not the standard of 35 U.S.C. § 103. *In re Goodwin*, 198 USPQ 1, 3 (CCPA 1978); *In re Antonie*,195 USPQ 6 (CCPA 1977); *In re Tomlinson*, 150 USPQ 623 (CCPA 1966).

The Examiner has not responded to the substance of this argument but instead says "the issue at hand is not whether other "prior art" references may have suggested a fusion protein lacking a linker, but rather whether the prior art cited as the basis of this rejection teaches or suggests a fusion protein comprised of such a linker." (Office Action Page 14) This is a plainly erroneous statement of both the law and the facts. The issue is in this case is whether the prior art of record, taken as a whole, suggests the specific combination of a PSMA-scFV, a CD8 hinge and a cytoplasmic domain as claimed.

Furthermore, as Applicants have twice argued, the published Guest reference refutes any arguments of obviousness. The Examiner's failure to review the record and the documents in the record means that the arguments of the Examiner in the office action are totally off point. The Examiner's statement that "Applicants has not provided any logical or scientific basis supporting" an assertion that there would be no reasonable expectation of success (Office Action Page 12) is not supported by the complete record. The Guest reference supports Applicants contention that there the art is unpredictable, and that the Examiner may not use generalized teachings to suggest a particular combination.

Thus, the rejections under 35 USC § 103 should be withdrawn.

Claims 1-3, 12, 13, 25, 26, 29 and 30 stand rejected for obviousness-type double patenting in view, and as not being patentably distinct from US Patent Application No. 10/448,256 in view of the now cited Bebbington art. Applicants traverse both these rejections.

As previously observed, application Serial No. 10/448,256 claims priority to a provisional application filed May 28, 2002. This present application was published on March 16, 2000 as PCT Publication No. WO 00/14257. As such, the disclosure of the present application is prior art with respect to the cited application, and presumably claims will not issue in that case which are obvious over the disclosure of the present application. The Examiner asserts that this is irrelevant to the issue of double patenting. In doing so, he is in error. Double-patenting is a judicially created doctrine to prevent an inventor from avoiding his own earlier applications effects as prior art. In this case, the cited application is later in date than this one, and the disclosure of this application is effective as prior art. This being the case, the present double patenting rejection puts Applicants in a worse position that a third party would be. That is not what the doctrine was intended to do, and therefore this application of it must be wrong.

Furthermore, the invention of the cited application is different from that now claimed. As previously explained:

(1) In the present application, the claims are directed to a fusion which includes a cytoplasmic domain, such as the CD3 zeta-chain cytoplasmic domain **or** the CD28 cytoplasmic domain.

(2) The claims of the cited application are to a fusion which includes the intracellular domain of the CD3 zeta chain **and** a costimulatory region such as the intracellular domain of CD28.

(3) Applicants recognize that a fusion within the scope of the claims of this case **could** have both intracellular domain and costimulatory region because the claims are in comprising terms.

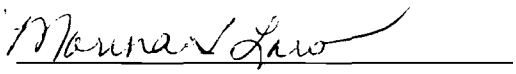
(4) This means that a single construct could fall within the claims of both applications (assuming it met all the other limitations). This is why Applicants said that the claims of this application could "dominate" the claims of the other application.

(5) Domination alone is not a basis for an obviousness-type double patenting rejection. The Examiner must also show obviousness.

In this case, because the present disclosure is prior art with respect to the cited application, the proper forum for a determination of the obviousness of the cited application vis a vis the present invention is in the prosecution of the cited application. There, the Examiner may apply the full disclosure of the present invention and any secondary references that are deemed applicable, consider evidence of unexpected results, and make a determination. As noted above, however, there is simply no reason to seek a terminal disclaimer in this case since none of the reasoning underlying the doctrine of obviousness-type double patenting applies. To do so, would turned it from an equitable doctrine into an inequitable one.

For these reasons, the elected claims are believed to be in form for allowance. Accordingly, recombination of the withdrawn claims related to unelected species and methods of using the fusion proteins and allowance of the application as a whole is hereby requested.

Respectfully Submitted,



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# The Role of Extracellular Spacer Regions in the Optimal Design of Chimeric Immune Receptors

## *Evaluation of Four Different scFvs and Antigens*

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**Summary:** Human peripheral blood lymphocytes can be transduced to express antigen-dependent CD3ζ chimeric immune receptors (CIRs), which function independently of the T-cell receptor (TCR). Although the exact function of these domains is unclear, previous studies imply that an extracellular spacer region is required for optimal CIR activity. In this study, four scFvs (in the context of CIRs with or without extracellular spacer regions) were used to target the human tumor-associated antigens carcinoembryonic antigen (CEA), neural cell adhesion molecule (NCAM), the oncofetal antigen 5T4, and the B-cell antigen CD19. In all cases human T-cell populations expressing the CIRs were functionally active against their respective targets, but the anti-5T4 and anti-NCAM CIRs showed enhanced specific cytokine release and cytotoxicity only when possessing an extracellular spacer region. In contrast, the anti-CEA and anti-CD19 CIRs displayed optimal cytokine release activity only in the absence of an extracellular spacer. Interestingly, mapping of the scFv epitopes has revealed that the anti-CEA scFv binds close to the amino-terminal of CEA, which is easily accessible to the CIR. In contrast, CIRs enhanced by a spacer domain appear to bind to epitopes residing closer to the cell membrane, suggesting that a more flexible extracellular domain may be required to permit the efficient binding of such epitopes. These results show that a spacer is not necessary for optimal activity of CIRs but that the optimal design varies.

**Key Words:** T lymphocytes, T-cell receptors, gene therapy, cellular activation, tumor immunity

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Improved treatments for cancer are needed, and the use of tumor-specific T cells as a therapy against cancer is attractive. Experimental[1,2] and some clinical data[3,4] suggest that they can be effective, but it is also clear that many tumors have evolved to evade the immune system.[5] There are several mechanisms for this: (1) the effects of factors produced by the tumor that are deleterious to T-cell survival[6]; (2) the down-regulation of key immune regulatory molecules (including MHC molecules)[5,7]; and (3) the fact that the majority of tumors develop spontaneously and as such express largely "self-antigens" against which T cells are immunologically tolerant. In the clinical situation, antigen-specific T cells may thus fail to function effectively due to the harsh tumor microenvironment, or alternatively the tumor may be effectively invisible to T cells through a lack of recognition molecules present on the surface of the tumor.

To address some of these issues and to facilitate the generation of large numbers of antigen-specific T cells, the concept of redirecting T-cell activity using antibody technology has been developed. Chimeric immune receptors (CIRs) on the surface of the T cell are activated by recognition and binding of the appropriate cell surface antigen on the target cells. Antibody-based receptors, first described by Eshhar et al, consist of antigen-binding domains fused to the signaling domains of key immune receptors.[8] Antigen recognition and activation occur directly as a result of the CIR binding protein antigen, and hence target cell killing occurs independent of TCR binding of the MHC/peptide complex.[8] The most commonly used CIRs comprise a single-chain variable fragment (scFv) antigen-binding domain fused to either the CD3ζ or Fcγ receptor.[8,9]

T cells expressing a variety of CIRs have been shown to induce antigen-dependent target cell lysis,[8 22] but the structure of the CIRs has varied. Work targeting the HER-2/Neu (Erb-2) receptor clearly showed that optimal T-cell responses were elicited only when a spacer domain consisting of hinge regions was incorporated into the CIR.[10] Other studies have used various spacer domains with CIRs to target a diverse range of protein molecules, including HIV gp120,[14] CD20,[20] CEA,[11,12] and TAG-72[18]; however, comparative data with receptors lacking spacer domains have not been reported. Examples of the spacer regions used include immunoglobulin domains (either the Fc region of IgG1 or the immunoglobulin-like extracellular regions of CD4 or CD8) and CD4 or CD8

transmembrane domain.[8–22] Interestingly, our recent work targeting carcinoembryonic antigen (CEA) using the MFE23 scFv has shown that the CIR was effective at targeting CEA-expressing cell lines despite the absence of an extracellular spacer domain.[11,17] In view of this observation, our study sought to investigate the contribution of CIR structure and the role of a spacer to the efficiency of T-cell function. We compared the relative activity of CIR-modified T cells against multiple antigens to ascertain whether a correlation between the target antigen and CIR structure could be determined. Consequently, the scFvs used in these experiments specifically bound the tumor-associated antigens CEA, neural cell adhesion molecule (NCAM), B-cell antigen (CD19), and oncofetal antigen 5T4.[23–26] The basic structures of the antigens are quite different (Fig. 1).

## METHODS

### Cells and Antibodies

All cell culture media were obtained from Invitrogen unless stated otherwise. The cell lines 293T,[27] MKN45K (CEA and 5T4-positive human gastric carcinoma, JCRB No. 0254), LS174T (CEA and 5T4-positive human colon adenocarcinoma, ECACC No. 87060401), LoVo (CEA and 5T4-positive human colon adenocarcinoma, ATCC No. CCL-229), NCAM-positive SK-N-BE (human neuroblastoma, ATCC No. CCRL-2271), and Lewis lung carcinoma (ATCC No. CRL-1642) were all routinely cultured in DMEM Glutamax medium and 10% heat-inactivated fetal bovine serum. Raji B lymphoma cell line (ATCC No. CCL-86) was routinely grown in 90% RPMI 1640 buffered with HEPES (25 mM, Sigma), 2-mercaptoethanol (50 nM), L-glutamine (2 mM), and 10% heat-inactivated fetal bovine serum. Anti-human CD3 PE-conjugated (clone UCHT1), anti-human CD4 PE-conjugated (clone RPA-T4), anti-human CD8 PE-conjugated (clone RPA-T8), anti-human CD19 PE-conjugated (clone HIB19), and anti-human CD25 PE-conjugated (clone M-A251) monoclonal antibodies were purchased from PharMingen. The antibody used for Western blotting was mouse anti-human CD3ζ (clone 8D3, PharMingen) bound by a secondary anti-mouse (peroxidase conjugated, Sigma).

The scFv MFE23 is known to recognize the N-A1 region of CEA[28] and the LL/2 cell line was transduced to express these domains of CEA. The N and A1 domains of CEA were isolated from a human normal colon cDNA library using primers "Ndomain NcoI back" (5′-ACGTACTCGC GGCCCAACCG GCCATGGCCA AGCTCACTA TTGAATCC-3′) and "A1domain NotI forward" (5′-GATATGAGAT ACTGCGGCC GCATAGAGGA CATTCAGGAT GACTG-3′). The PCR fragment was initially cloned into a pUC119-based bacterial expression vector and subsequently into a retroviral vector for transduction of LL/2 cells. A clone stably expressing the CEA fragment was selected (see Fig. 1).

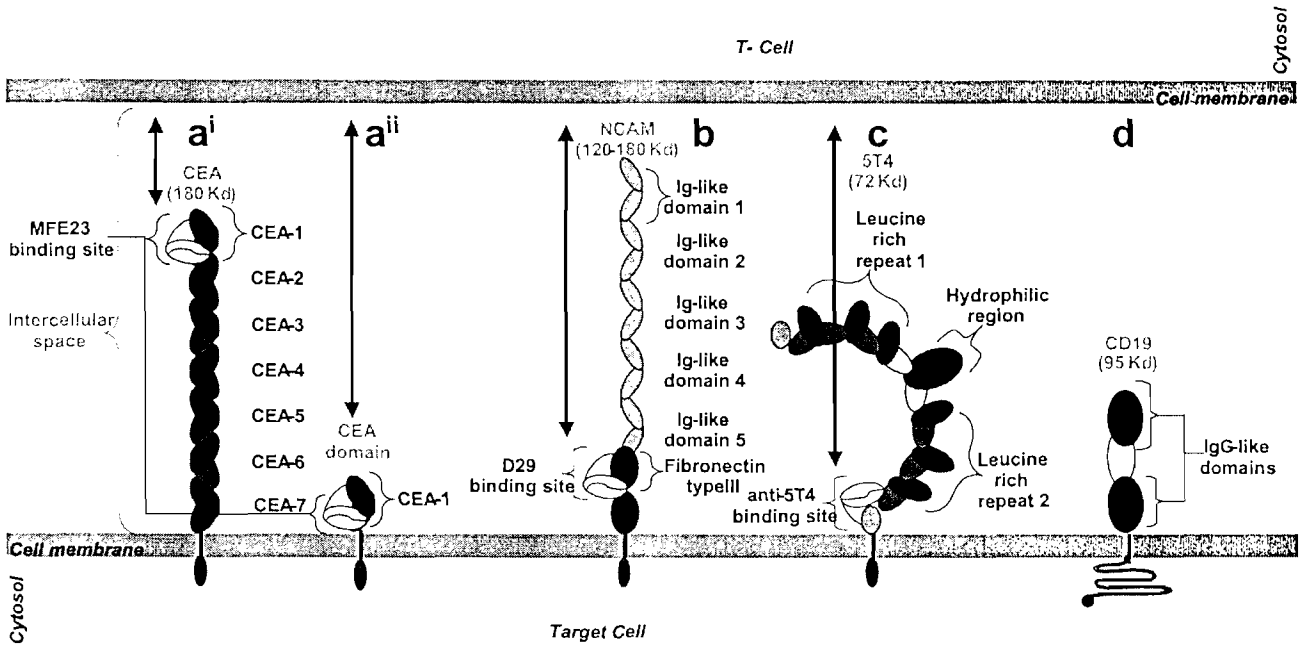


FIGURE 1. Tumor-specific antigens CEA, NCAM, 5T4, and CD19 and the scFvs binding domains where they have been characterized. The arrows indicate a possible difference between the specific epitope and the T-cell membrane. a$^{i}$, the MFE23 epitope on CEA is located away from the membrane, which would be close to the T-cell membrane[28]; a$^{ii}$, the modified CEA molecule expressing only the ScFv binding domain (CEA-1); b, the D29 epitope on NCAM is located close to the tumor cell, and therefore due to the large extracellular domain, the epitope is either buried or pushed away from the T-cell membrane[30]; c, the anti-5T4 epitope is also located close to the tumor cell membrane, possibly buried within the 5T4 molecule[38]; d, the CD19 molecule has been characterized, but as yet the binding domain of the antibody/scFv from the hybridoma HD37 is unknown.[26]

## ScFv and Fusion Proteins

The anti-CD19 scFv was isolated from the HD37 hybridoma[26] using mouse V-gene family specific primers as previously described.[29] The humanized scFv D29 was constructed from a monoclonal antibody against human NCAM.[24,30] The mouse scFvs against 5T4 and CEA (MFE-23) have been previously described[23,25] and were fused to human IgG1 Fc domains to produce "antibodies" for cell staining and were expressed using a CMV-driven expression vector. The resultant supernatant from 293T cells transfected with the vector was used undiluted to identify tumor-associated antigens upon target cells. Anti-human Fc (PE conjugated, Sigma) was used as the secondary antibody against the scFv fusion proteins: anti-5T4-hFc and MFE23-hFc.

## Construction of Chimeric Receptors and Retroviral Vectors

Two basic CIR formats were used (Fig. 2). The standard scFv.CD3ζ IRES GFP vector was constructed as previously described.[11] The spacer region (IgG1 Fc domain) was isolated from PBMC mRNA by RT-PCR using the following nucleotide primers: forward primer (containing a *Not I* site): 5′-ACTGTCCTCG-AGGAG**GCGGC-CGC**AAAATCT-TG TGAC-3′; reverse primer (containing a *Fse I* site): 5′-GG CTGATCAG-CGAGCTCT**GG-CCGGCC**CTTT-ACCCGG AGAC-AGGGAG-3 for the whole IgG1 Fc domain (CH2CH3). These were inserted between the scFv and the CD3-ζ to produce the construct with spacer regions. Retroviral vectors were prepared by cotransfection of 293T cells as previously described.[11,31]

## T-cell Isolation and Activation

Peripheral blood lymphocytes (PBLs) of healthy donors diluted 1:1 with T-cell medium were isolated by centrifugation (400*g*, 25 minutes at room temperature) through Histopaque (Sigma), washed twice and resuspended in T-cell culture medium (AimV [Invitrogen], with 2% pooled AB serum) prior to depleting monocytes by plastic adherence. The nonadherent cells were activated on non-tissue culture plates coated with mouse anti-human CD3ε (1 μg/mL, Orthoclone OKT-3, Orthobiotech) and mouse anti-human CD28 (1 μg/mL, clone 37407.111, R&D Systems) at a density of $0.5 \times 10^6$ cells/mL along with rIL-2 (100 IU/mL, Amersham). Lymphocytes were cultured in T-cell culture medium at a density of $0.5 \times 10^6$ cells/mL along with rIL-2 (100 IU/mL) and maintained every 2 to 3 days.

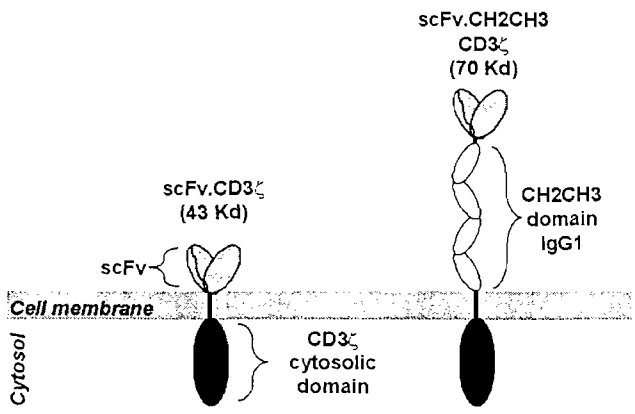


**FIGURE 2.** Expected structure of the CIR. The native CD3ζ was fused directly to the scFv (scFv.CD3ζ).[11] The second construct contains the hinge and CH2CH3 domain (scFv.Fc.CD3ζ) from IgG1 fused between the scFv and the CD3ζ chain.

## Transduction of Retroviral Vectors

Lymphocytes activated for 3 days were washed and resuspended in viral supernatant at $0.2 \times 10^6$ cells/mL, 6 μg/mL Polybrene (Sigma), and rIL-2 (100 IU/mL). The cells were centrifuged at 1,500*g* for 3 hours at room temperature before being washed and resuspended ($0.5 \times 10^6$/ml) in T-cell culture medium supplemented with rIL-2 (100 IU/mL). The process was repeated 24 hours later with a second batch of viral supernatant, before expansion in T-cell culture medium supplemented with rIL-2 (100 IU/mL). Prior to any assays (2–3 days), the cells were centrifuged and resuspended in fresh medium along with low rIL-2 (20 IU/mL). All assays were carried out within a 2-week period after transduction to identify a clinically relevant response. To produce comparative levels of transduction, the percentage of cells bicistronically expressing GFP was assumed to be a good indicator of the level of transduction and hence the percentage of cells expressing the CIR. Comparison of the MFI of GFP within scFv-specific CIRs showed little difference and the level of intensity did not correlate with overall response. Therefore, prior to all experiments the transduced T cells were analyzed by flow cytometry for GFP and the cells were diluted with mock transduced cells to give equivalent transduction frequencies (as measured by %GFP). Due to the variation in transduction efficiency, the percentage of mock cells in the final assay mixture varied from 75.7% to 0% (mean 25.3 ± 24.1). Furthermore, there was no correlation between the response of a construct and the amount of dilution.

## Cytotoxicity Assay

The chromium-release assays were performed as described previously.[11] Briefly, transduced T cells ($1 \times 10^5$ to $3.125 \times 10^4$ cells/well) were added in triplicate to 96-well microtiter plates (100 μL/well). The target cells were labeled with 100 μCi $^{51}Cr$ (sodium chromate, ICN) per $5 \times 10^5$ cells for 2 hours at 37°C, and then 5,000 target cells (100 μL) were added and incubated for 8 hours (37°C, 5% $CO_2$). After the 8-hour incubation, 100 μL of supernatant was collected from each well and dried on Luma plates (Packard) overnight before counting on a Microplate Scintillation Counter (Packard). Percentage specific lysis was calculated as follows: ([experimental counts − spontaneous counts]/[maximum counts − spontaneous counts]) × 100.

## Direct Antigen Activation Assay

Non-tissue culture 96-well microtiter plates (flat-bottomed) were coated with borate buffer alone (0.1 M boric acid, 0.15 M NaCl, pH 8.5), CEA (Sigma), h5T4.hFc,[25] or NCAM[24] at a concentration of 1 μg/mL for 2 hours at 37°C or overnight at 4°C, followed by a PBS wash and T-cell culture

medium blocking for 20 minutes. The T cells were added, in triplicate sets, at $1 \times 10^5$ cells/well without IL-2 and incubated for 3 days (37°C, 5% $CO_2$). Medium (100 μL) was removed for cytokine analysis.

### IFNγ ELISA

The antibodies and cytokine used for ELISA were all from R&D Systems (UK). The capture antibody was mouse anti-human IFNγ (clone 25718.111). The detection antibody was biotinylated mouse anti-human IFNγ (clone 25723.11). The matched pair antibodies were used according to the manufacturer's instructions. To quantify the ELISA, IFNγ standards were added in triplicate to produce a standard curve (2,000 pg/mL to 31.25 pg/mL).

### Flow Cytometry

The membrane expression of CD3, CD4, CD8, and CD25 on isolated PBLs was measured by flow cytometry on a FACScan flow cytometer (Becton Dickinson). Cells were washed twice in PBS containing 2% BSA (PBS/BSA) and resuspended in 100 μL PBS/BSA containing the relevant PE-conjugated antibody. The cells were incubated with antibody for 30 minutes at 4°C in the dark. After incubation, cells were washed twice (PBS/BSA) and resuspended in 300 μL of 1% paraformaldehyde. Treatment with the relevant nonspecific PE-conjugated antibody served as a staining control.

### Western Blotting

Cells were harvested, washed, and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.5% SDS, and 50 mM Tris-Cl, pH 8.0). Equivalent numbers of cells were separated by SDS-PAGE on 10% resolving gels by standard methods and subsequently blotted onto PVDF membrane (Roche). Membranes were probed with a 1:1,000 dilution of anti-CD3ζ Mab (PharMingen) with a secondary of HRP-labeled sheep anti-mouse IgG (1:10,000, Sigma) followed by visualization using ECL (Amersham).

### Statistical Analysis

The Student *t* test was used to determine whether two samples were likely to have come from the same two underlying populations using a paired two-tailed analysis. A significant result was determined as $P = 0.05$.

## RESULTS

### Expression of Chimeric Immune Receptors in Polyclonal T-Cell Populations

Retroviral vectors were generated that encoded the various scFv-CD3ζ vectors and were then used to transduce primary human T lymphocytes. Two classes of receptors were generated. The first set contained the scFv directly fused to the CD3ζ[11] and the second set was engineered to contain an extracellular spacer region comprising the hinge and CH2CH3 domains (Fc) of human $IgG_1$ between the scFv and CD3ζ domains. The predicted structure of the receptors used is shown in Figure 2.

After transduction and expansion in IL-2 (in the absence of selection for the transduced cells), Western blot analysis confirmed that the CIRs were expressed to their predicted molecular weight (Fig. 3A). Constructs possessing the Fc spacer region showed some sign of proteolysis of the receptor as identified by a faint immunoreactive band of approximately 45 kDa. This was a consistent observation in all Fc receptors, but the full-length protein was expressed at far higher levels in all cases (see Fig. 3A and data not shown).

Analysis of the phenotype of the T-cell populations (4–7 days after transduction) confirmed that the majority of the cells were CD3-positive T cells (96 ± 3.4%); the majority of the cells were CD8 positive (76 ± 5.3%), with CD4 T cells representing the remainder (27 ± 4.7%). The level of gene expression within either the CD4 or CD8 population remained consistent with the level of overall CD3 transduction. The cells were analyzed for retroviral transduction using the reporter gene GFP, which was expressed bicistronically with the CIRs via an IRES element.[11] In addition, the level of MFE23 CIR surface expression was determined by labeling the cells with biotinylated-CEA (bCEA) and streptavidin-PE (see Fig. 3B). An approximate linear relationship between the intensity of GFP expression with the degree of bCEA labeling was observed for both constructs with or without the extracellular spacer. For subsequent experiments, the level of GFP expression was therefore used as a measure of CIR expression. To compensate for variability due to differences in T-cell transduction frequency with the various constructs, the T-cell populations were adjusted to express the same relative proportion of transduced T cells, as assessed by GFP expression, by the addition of T cells from the same donor that had been mock-transduced and expanded under identical conditions.

### An Extracellular Spacer Domain Enhances the Activity of T-Cell Populations Expressing Either Anti-5T4 or Anti-NCAM CIRs

Polyclonal T-cell populations transduced to express the anti-5T4 CIRs were tested for functional activity. In each case, T-cell populations specifically released IFNγ upon culture with recombinant 5T4 protein and cell lines known to express 5T4 only when expressing the anti-5T4 receptors, while mock-transduced T-cell populations produced low background levels of cytokine (Fig. 4A). Similarly, high specific levels of IFNγ release were detected only from T-cell populations expressing the anti-NCAM D29.CD3ζ CIR when cultured with recombinant NCAM protein or the NCAM-expressing cell line SK-N-BE (see Fig. 4B). This experiment confirmed that the CIRs were redirecting T-cell effector function in the absence of normal TCR restriction, since the T-cell populations were reacting against recombinant proteins immobilized on culture plates. It was also clear that for each target, the CIR that possessed the extracellular Fc domain generated the greater degree of cytokine release compared with constructs where the scFv was fused directly to the CD3ζ moiety. This difference was also reflected in the cytotoxicity assay, where an increased cytotoxic potential was observed for the anti-5T4 (see Fig. 4C) and anti-NCAM (see Fig. 4D) CIR-expressing T cells that contained the extracellular spacer. The results with both anti-5T4 and anti-NCAM receptors clearly showed that the presence of an extracellular spacer enhances the activity of

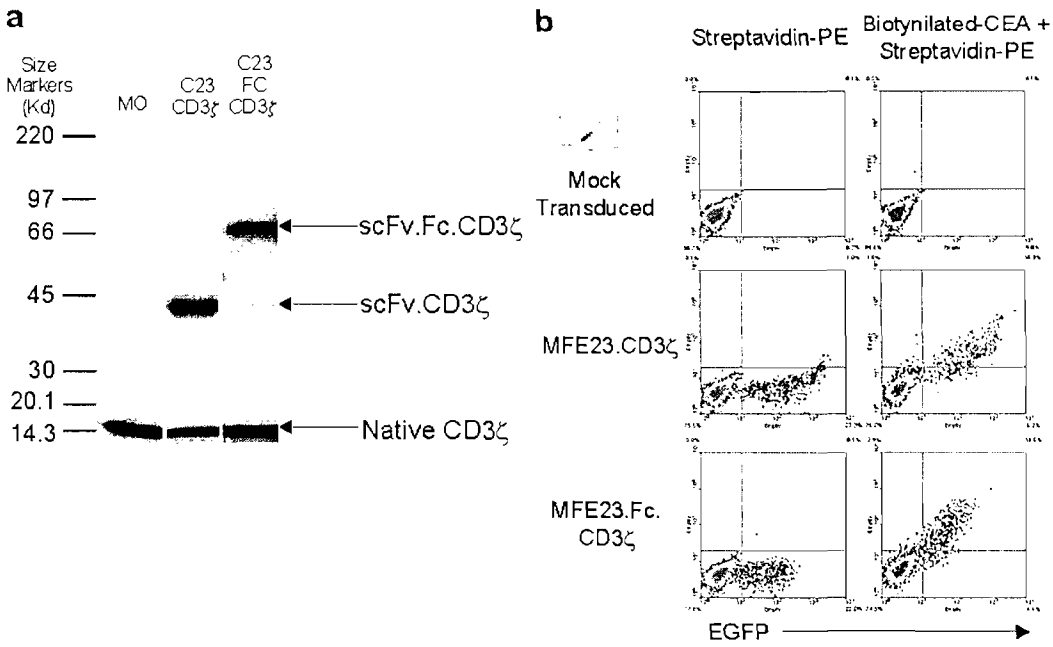


**FIGURE 3.** Analysis of transduced T-cells with MFE23-based CIRs. A, To assess CIR expression, transduced T cells were analyzed for protein expression by Western blotting. Equivalent numbers ($2 \times 10^6$ cells/lane) of T cells from each culture were lysed and separated on an 8% denaturing SDS-PAGE gradient gel. The Western blots were probed with a mouse anti-CD3ζ mAb and a secondary anti-mouse IgG-peroxidase conjugate Ab. The reducing conditions blot shows the monomeric form of CIR and native CD3ζ (16 kDa). B, Transduction and expression efficiency of unselected T cells using GFP and surface expression of the respective MFE23 CIRs. All cells in the live gate were analyzed for GFP expression. The prefix MFE23 (anti-CEA-specific scFv) refers to the specific scFv in each CIR. The mock, MFE23.CD3ζ, and MFE23.Fc.CD3ζ density charts are shown to illustrate GFP-negative cells. The graph shows the percentage of GFP-expressing cells for each transduced population (streptavidin-PE) and surface expression of the CIR (biotinylated-CEA and streptavidin-PE). These FACS plots show a close correlation between CIR expression and the reporter gene eGFP.

CIR-expressing T cells in an in vitro assay, as previously reported by others.[21]

## The Presence of an Extracellular Spacer Reduces the Magnitude of MFE23 CIR-Driven T-Cell Responses

To assess the efficiency of the anti-CEA CIRs against their specific antigen, the modified T-cell populations were tested for cytokine production in response to coculture with plate-bound CEA protein (Fig. 5A). T-cell populations transduced with the MFE23.CD3ζ generated high levels of IFNγ, while control T-cell populations produced only background levels of cytokine. In contrast to the results obtained with the anti-5T4 and anti-NCAM CIRs, significantly ($P = 0.002$) less IFNγ was produced when the CIR possessed an Fc domain compared with the spacer-less CIR. Importantly, equivalent levels of IFNγ were produced from all three populations when cultured with a polyclonal activatory antibody, OKT3 (data not shown). This effect was maintained when the T-cell populations were cultured on CEA-expressing tumor cell lines. Significantly ($P = 0.001$) greater levels of IFNγ were produced when the CIR lacked an extracellular spacer, indicating that this effect was independent of whether the protein was presented on plastic or on a cell surface (see Fig. 5B).

A standard chromium-release assay was then used to assess whether the difference in IFNγ secretion correlated with the potency of the modified T cells to lyse the CEA-expressing MKN45-K cell line (see Fig. 5C). Interestingly, there was no significant difference in CEA-targeted cytolytic activity between T-cell populations expressing CIRs that either possessed or lacked the extracellular Fc regions. These results were corroborated using other CEA-expressing tumor lines (data not shown), while the T-cell populations were further assessed for killing activity against CEA-negative target cells to confirm that there was no difference in the nonspecific cytolytic activities of the T-cell populations (data not shown). These observations suggest that in the case of the MFE23 scFv targeting, an extracellular spacer region does not enhance the activity of the CIR. However, it is also clear that there appears to be a degree of separation in downstream responses to CIR ligation with target antigen, or a spacer region linked to the MFE23 CIR clearly affects the IFNγ response circuit while having little effect upon the cytotoxic activity of the T cells.

To investigate this effect further, a murine tumor cell line LL/2 was transduced to overexpress the MFE23 scFv binding epitope of CEA(23) (see Fig. 1) and was used as a target cell line in a cytotoxicity assay. Once again, no clear difference in targeted CEA cytotoxic potential of the MFE23-based CIRs

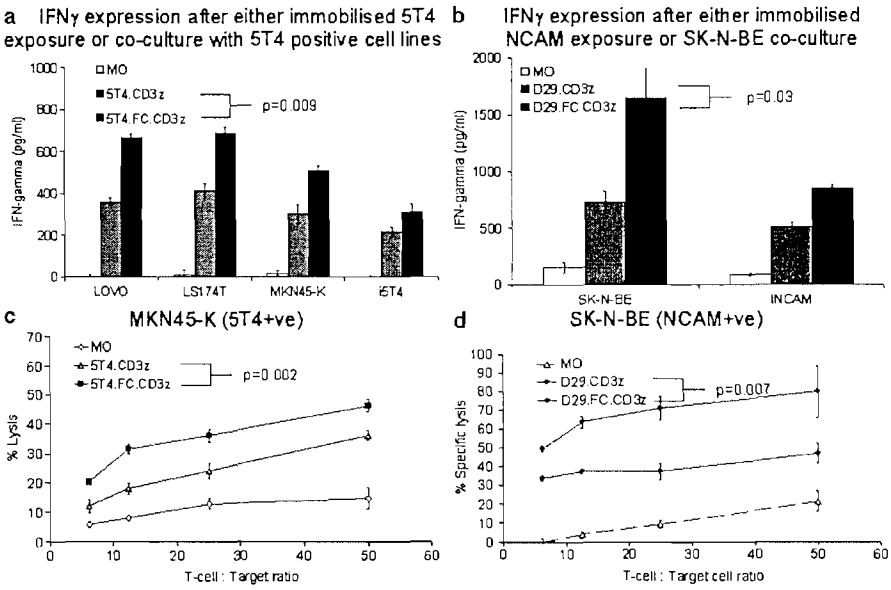


FIGURE 4. An Fc spacer domain in both the 5T4 and NCAM-specific scFvs (5T4 and D29 respectively) enhanced chimeric receptor function. Unselected polyclonal T cells (99% CD3 positive as determined by FACS analysis) from at least three donors were tested for their ability to secrete IFNγ after exposure to either immobilized antigen or antigen-expressing tumor lines and were also assessed for their cytotoxic activity upon antigen-expressing cell lines (results shown are from one example of at least three donors with SD from n = 3 replicates). A, Medium from T cells cultured with LOVO, LS174T, MKN45-K, or immobilized 5T4 (i5T4) precoated plates was analyzed for IFNγ by ELISA release after 24 hours. Only T cells transduced with 5T4 targeted CIRs induced IFNγ release upon culture with the 5T4-positive cell lines and i5T4. Insertion of an Fc spacer domain enhanced IFNγ expression significantly for both the cell lines and i5T4. B, Insertion of a spacer domain significantly enhanced IFNγ secretion when D29-based CIRs were co-cultured with the NCAM-expressing cell line SK-N-BE or iNCAM. C, in vitro cytotoxicity assays of 5T4-based CIR-expressing T-cell populations incubated for 8 hours with $^{51}Cr$ prelabeled MKN45-K. Although both T-cell populations of 5T4-targeted CIRs efficiently lysed the MKN45-K cell line, insertion of a spacer domain significant enhanced the cytotoxic activity of the CIR. D, Similarly, insertion of a spacer domain significantly improved the ability of D29-based CIRs to lyse the NCAM-expressing cell line SK-N-BE.

was observed between the T-cell populations (see Fig. 5D). Furthermore, the same cell line was used to compare the ability of MFE-based CIRs to induce IFNγ. As before, the insertion of the Fc spacer domain significantly reduced the ability of the MFE-based CIR to induce IFNγ expression, indicating that the absence of the majority of the CEA protein did not result in the requirement for a spacer to produce optimal CIR signal.

## CD19 Targeting CIRs Do Not Require an Extracellular Spacer Domain for Optimal Activity

The human CD19 B-cell antigen is a 95-kDa transmembrane glycoprotein and is considered to be a good tumor target due to its consistent expression across the majority of B-cell malignancies.[32] CD19 expression is limited to pre-B and B cells, is not expressed on hemopoietic stem cells, and is not shed, making it a goal for antibody-based therapy.[33] A scFv specific for human CD19 was generated (from the HD37 hybridoma[26]), and CIR constructs containing this scFv were used to test for their ability to target CD19-expressing cells. When human Burkitt's lymphoma Raji cell lines expressing CD19 were used as targets, it was clearly evident that a spacer domain significantly reduced the level of IFNγ expression generated by T cells in response to antigen (Fig. 6A). However, similar to the situation observed with the MFE23-based CIRs, no significant difference in targeted lysis was observed between spacer-containing or spacer-less anti-CD19 CIRs when transduced T cells were co-cultured with Raji cells in cytotoxicity assays (see Fig. 6B).

## DISCUSSION

The effector functions of T lymphocytes are mobilized as a result of the specific interaction of the TCR with MHC/peptide complexes present upon target cells. Considerable effort has been focused on understanding the sequential events and optimal requirements for full T-cell activation.[34] However, chimeric receptor technology has allowed the manipulation of T-cell responses to be directed under the control of antibody targeting, thereby avoiding the need for TCR-MHC/peptide interactions. Numerous studies have clearly shown that T cells expressing CIRs respond to protein antigen by cytotoxicity, cytokine production, and proliferation, all of which are key components of any potential immune therapy. However, few studies have investigated the basic structural requirements for optimal CIR activity and whether this differs for varying antigens. One study showed that an extracellular spacer domain was required for maximal response in CIRs directed against the HER-2/neu antigen.[21] We have investigated receptors targeted to four antitumor antigens. Similarly to the previously reported results, two of the CIRs targeted against the tumor antigens 5T4 and NCAM required an extracellular Fc domain to generate a maximal response in terms of cytotoxicity and IFNγ release. In contrast, the situation with respect to IFNγ release was reversed in the case of the CIRs used to target CEA and CD19. In both of these CIRs, the presence of an extracellular spacer resulted in reduced IFNγ release from the gene-modified T cells.

Although there was no reduced cytotoxicity in vitro, a number of studies have illustrated the importance of IFNγ secretion for tumor-specific T cells in tumor rejection and show that tumor rejection correlates well with IFNγ

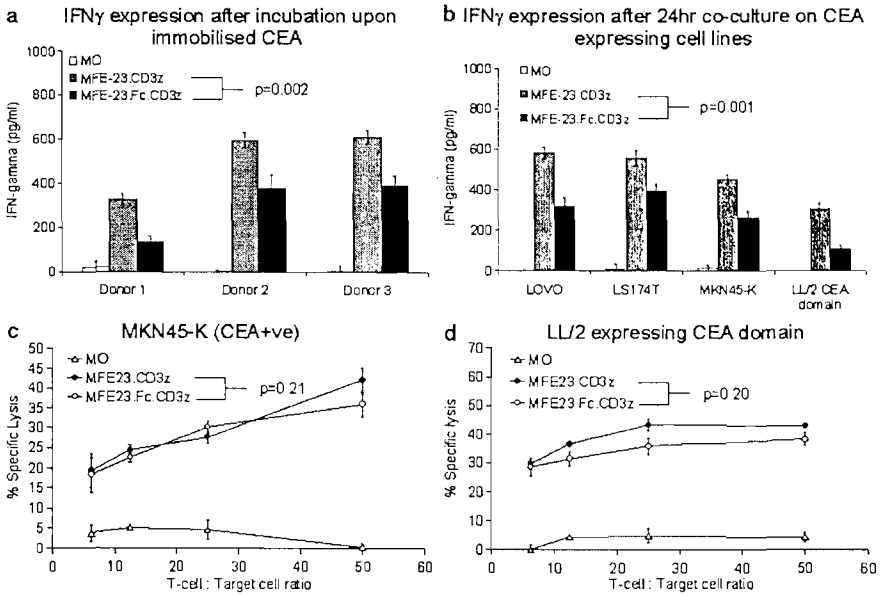


**FIGURE 5.** Polyclonal T cells expressing MFE23-based CIRs were exposed to CEA either immobilized or expressed upon the surface of cell lines to observe their ability to induce either IFNγ or lysis of antigen-expressing cell lines (from at least three different normal donors with the SD from n = 3 replicates). A, MFE23-based CIR transduced T-cell populations from three different donors were assessed for their ability to express IFNγ after immobilized antigen-dependent stimulation. After 3 days on immobilized CEA precoated plates the transduced T cells were specifically activated to release IFNγ compared with the untransduced mock cells. Furthermore, insertion of a spacer domain (Fc) significantly reduced the ability of the T cells to secrete cytokine. B, IFNγ release was also assessed when the T cells were incubated with CEA-expressing tumor cell lines, and once again inclusion of a spacer domain significantly reduced expression. LL/2 truncated CEA refers to a mouse cell line that has been transduced to express a protein encoding just the MFE23 scFv binding epitope of CEA attached to a transmembrane domain. C, To assess the lytic ability of the transduced populations, the T cells were co-cultured for 8 hours with $^{51}$Cr prelabeled MKN45-K cells. This revealed that although the T-cell populations expressing MFE23-based CIRs could effectively lyse the MKN45-K cells, there was no real difference between the CIRs with or without a spacer. D, T-cell populations were co-cultured for 8 hours with $^{51}$Cr prelabeled LL/2 cells expressing a truncated CEA protein (see Fig. 1A(ii)). This revealed that although the T-cell populations expressing MFE23-based CIRs could effectively lyse the truncated CEA-expressing cells, there was no real difference between the CIRs with or without a spacer. This suggests that the overall space between the tumor cell and T cell may not be critical to function of the CIR.

production.[32,35,36] There is also evidence to suggest that the strength of TCR signaling directly affects cytokine signaling, with a strong TCR signal required for IFNγ secretion.[36,37] Studies using a CIR specific for the renal carcinoma cell antigen G250 clearly showed that cytokine production and cytotoxicity were dependent on the levels of antigen expression on target cells and the levels of receptor expression upon the effector cells.[15] In this report, T cells expressing low levels of CIR on the cell surface responded only against target cells expressing high levels of antigen. However, T cells with high levels of CIR responded equally to target cells expressing low or high amounts of target antigen.[15] In our study, the differences in T-cell response were generated through the presence or absence of an extracellular spacer region. A further possible explanation to account for these differences could be variations in the level of CIR expression between the T-cell populations. However, flow cytometry for the bicistronic GFP marker gene indicated that the degree of transduction between groups was equivalent (see Fig. 3B, and data not shown). Furthermore, Western blot analysis showed broadly equivalent levels of total CIR protein expressed by the T cells targeted against CEA (see Fig. 3A, B).

These results suggest that other factors may be at work to account for the observed functional differences in T-cell response to antigen. It appears that IFNγ secretion is directed by a strong CIR-mediated signal.[36] In the 5T4 and NCAM-based CIRs, an extracellular spacer region is required to generate a strong signal, while in the MFE23 and CD19-based CIRs, an extracellular spacer region is detrimental to the ability of the CIR to induce cytokine production. The binding sites for three of the scFvs have been mapped (see Fig. 1). Interestingly, in the case of MFE23, the binding site of the scFv resides near to the extreme amino-terminal of CEA, within the first truncated CEA (labeled as CEA-1[28]). In the case of both D29 (anti-NCAM) and anti-5T4 scFvs, the epitope binding sites reside much closer to the cell membrane. For D29, the epitope resides within the fibronectin type III binding site,[30] while the 5T4 scFv recognizes the membrane proximal part of leucine-rich repeat 2.[30] The epitope for MFE23 thus appears to reside a distance away from the cell membrane, while the epitopes for both NCAM and 5T4 reside much closer to the cell membrane.

One hypothesis that could fit these observations is that there is an optimal distance between T cell and target cells, and that where the target epitope for a CIR is far from the cells (as with MFE23), a spacer is not required. Conversely, when the epitope is very close to the membrane (eg, the epitopes on 5T4 or NCAM in these experiments), the absence of spacer regions in the anti-NCAM and anti-5T4 receptors may result in insufficient spacing to permit optimal T-cell activity. To examine this hypothesis, so that it was possible to manipulate the position of the target epitope relative to the cell membrane, a cell line that expressed a truncated form of CEA (see Fig. 1) was generated. This positioned the MFE23 epitope closer to the cell membrane; however, no difference in cytotoxic activity between the MFE23 CIRs either with or without the extracellular spacer domain was observed (see Fig. 5D). In addition, the ability of the MFE23-based CIRs to induce IFNγ

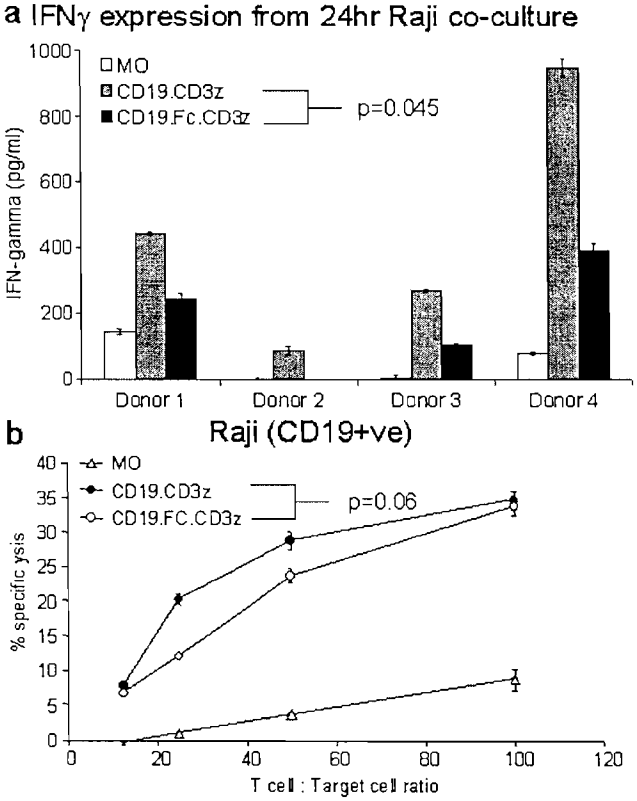


**FIGURE 6.** The antigen CD19 expressed on the Raji tumor cell line was able to induce activation-dependent IFNγ release and cytolysis (results shown are from one example of four donors with SD from n = 3 replicates). A, IFNγ secretion from co-culturing T cells transduced with anti-CD19-based CIRs from four different donors with CD19-expressing tumor cells (Raji). The results show an increase in IFNγ expression when the T cells were transduced with anti-CD19 CIR compared with the mock control. Furthermore, the insertion of a spacer region (Fc) significantly reduced the ability of the T cells to induce IFNγ (SD from n = 3 replicates). B, The T-cell populations were also tested for their ability to lyse $^{51}Cr$ prelabeled Raji cells. Although there was a significant difference between the mock and anti-CD19 CIR transduced cells, the insertion of the Fc domain had no effect (results shown are from one example of four donors with SD from n = 3 replicates).

when the T cells were exposed to the LL/2 cell line expressing the truncated CEA remained greatly reduced when the Fc spacer domain was incorporated (see Fig. 5B). Although the truncated CEA positioned the MFE23 epitope "closer" to the plasma membrane of the target cell, the context of the epitope was maintained in the fact that the domain of CEA remained as the amino-terminal end of the recombinant protein.

Thus, an alternative hypothesis, that the spacing between the T cell and its target may not be the principal constraining factor, but that the accessibility of the epitope may be more important, fits better with these observations. In the case of the anti-NCAM and anti-5T4 scFvs, a spacer region may be important in providing flexibility to allow access to epitopes that are relatively inaccessible close to the target cell plasma membrane. However, in the case of MFE23 targeting CEA, the epitope may be more accessible, and as such a flexible spacer domain would therefore be unnecessary for optimal ligation of CIR with target epitope. Having a flexible spacer may actually reduce the efficiency of the CIR compared with a rigid structure, where the epitope is easily located.

In summary, we have examined chimeric receptors against four different antigens and found that the optimal construct does not always require a spacer. It appears that the site of an epitope on a protein may contribute to the structural requirements for a CIR to generate optimal activity. These observations indicate the importance of testing various constructs (with and without a spacer) to obtain optimal chimeric receptor activity for a given antibody/antigen.

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